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Increased Proliferation of Osteoblastic Cells Expressing the Activating $G_s\alpha$ Mutation in Monostotic and Polyostotic Fibrous Dysplasia

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We studied the osteoblastic abnormalities resulting from activating mutation of the $G_s\alpha$ gene in two patients with McCune-Albright syndrome and one patient with monostotic fibrous dysplasia. Histomorphometric analysis of dysplastic lesions showed a low number of differentiated osteoblasts along the bone surface and numerous immature alkaline phosphatase-positive mesenchymal cells actively depositing a woven bone matrix. Osteoblastic cells isolated from dysplastic bone lesions expressed a missense mutation in the $G_s\alpha$ gene in position 201 and showed increased intracellular basal cyclic adenosine 3′,5′-monophosphate levels compared with normal cells isolated from a noninvolved area in the same patient. Cell proliferation evaluated by DNA synthesis was twofold to threefold greater in osteoblastic cells expressing the mutation compared with normal cells from the same patient and was greater in cells isolated from more severe than less severe fibrotic lesions. In contrast, the synthesis of osteocalcin, a marker of mature osteoblasts, was lower in osteoblastic cells expressing the $G_s\alpha$ mutation compared with normal cells from the same patient and was lower in cells isolated from severe compared with less severe fibrotic lesions, indicating that the increased growth in mutated osteoblastic cells was associated with reduced cell differentiation. The results show that activating mutation of $G_s\alpha$ in osteoblastic cells leads to constitutive activation of adenylate cyclase, increased cell proliferation, and inappropriate cell differentiation, resulting in overproduction of a disorganized fibrotic bone matrix in polyostotic and monostotic fibrous dysplasia. (Am J Pathol 1997, 150:1059–1069)

The McCune-Albright syndrome (MAS) is a sporadic disorder characterized by café-au-lait skin lesions, polyostotic lesions of fibrous dysplasia, and multiple endocrinopathies, such as sexual precocity, hyperthyroidism, pituitary adenomas secreting growth hormone, and autonomous adrenal hyperplasia.[1,2] Activating missense mutations of the gene encoding the α subunit of G_s, the G protein that stimulates cyclic adenosine 3′,5′-monophosphate (cAMP) formation, have been identified in sporadic human growth hormone-secreting pituitary adenomas and thyroid tumors[3–8] and in various tissues in MAS,[6,7] in particular bone.[9,10] Substitution of Arg^{201} with either Cys or His leads to abnormal $G_s\alpha$ protein, inhibition of the guanosine triphosphatase activity, and constitutive activation of adenylate cyclase,[3,6,7,11] resulting in increased cell proliferation in pituitary tumors.[4,8] The reported activating somatic mutations of the $G_s\alpha$ gene are thus believed to be responsible for the abnormalities observed in several affected tissues in MAS.[6,7]

Bone lesions found in monostotic and polyostotic fibrous dysplasia are characterized by woven ossified tissue, increased bone matrix formation, and extensive marrow fibrosis,[9,10,12] suggesting abnormalities in bone-forming cells. We recently reported the presence of activating somatic mutations of the $G_s\alpha$ gene in osteoblastic cells derived from fibrotic lesions in patients with monostotic fibrous dysplasia,[13] suggesting that the mutation may induce abnormal osteoblastic cell proliferation or function in this disorder. Mutational activation of the α-subunit

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has been associated with increased proliferation in a number of endocrine tissues and in fibroblasts.[4,14,15] It is, therefore, conceivable that mutations of the $G_s\alpha$ gene may induce abnormalities in the control of osteoblast growth and/or differentiation, resulting in fibrous dysplasia.

In this study, we analyzed the cellular events resulting from the activating mutation of $G_s\alpha$ in osteoblastic cells isolated from patients with MAS and with monostotic fibrous dysplasia compared with normal cells from the same patients. We report here that the activating $G_s\alpha$ mutation in monostotic and polyostotic fibrous dysplasia is associated with an increased proliferation and inappropriate differentiation of osteoblastic cells.

Materials and Methods

Patients

We studied two patients with MAS and one patient with isolated monostotic bone fibrous dysplasia. Patient 1 was a 42-year-old man with classical features of MAS, including café-au-lait pigmentation, polyostotic fibrous dysplasia involving the skull, face, spine, rib, and femur, and hypersecretion of growth hormone with acromegaly.[16] Patient 2 was an 18-year-old man with MAS features, such as café-au-lait pigmentation and polyostotic fibrous dysplasia involving the cubitus, femur, and skull, and acromegaly with hypersecretion of growth hormone and prolactin. Patient 3 was a 23-year-old woman with isolated monostotic bone fibrous dysplasia affecting the left frontal bone since 8 years of age. She had no family history, endocrine abnormalities, or café-au-lait pigmentation and, therefore, no clinical features of MAS.[16]

In each patient, an operation was performed to resect a dysplastic lesion, allowing investigation of the bone lesions at the tissue, cellular, and molecular levels. In patients 1 and 3, bone samples from dysplastic and normal craniofacial areas were obtained. In patient 2, a dysplastic bone sample from the femur was obtained. This bone sample was separated into severely and less severely affected fibrotic areas on a morphological basis. All samples were processed separately for histology, osteoblast cell cultures, and mutation analysis.

Histological Analysis

For histological examination, the bone specimens were fixed in ethanol at 4°C and embedded undecalcified in glycol methacrylate.[17] Five-micrometer-thick sections were stained with Goldner trichrome to identify bone matrix-forming sites and bone cells. The calcified matrix was identified by von Kossa staining, and the cells were stained histochemically for alkaline phosphatase (ALP) activity.[17] A histomorphometric analysis was performed to examine the activity of bone formation in involved areas of fibrous dysplasia. For each bone sample, histomorphometric indices of bone formation were measured using an ocular integrator coupled to a microscope (Olympus, New Hyde Park, NY) using conventional methods.[18] The following indices were measured: the osteoid surface (percentage of bone surface covered with uncalcified bone matrix), the osteoblast surface (percentage of bone surface and percentage of osteoid surface covered with osteoblasts), the osteoclast surface (percentage of bone surface covered with osteoclasts), the osteoclast number per square millimeter of bone, and the fraction of bone tissue composed of woven bone.

Osteoblastic Cell Cultures

Osteoblastic cells were obtained from explants of dysplastic bone lesions and from adjacent normal bone in patients 1 and 3 and from two separate parts, severe and less severely affected areas, in a dysplastic bone lesion in patient 2. Osteoblastic cells were isolated as described previously.[19] Briefly, the bone samples were washed in phosphate-buffered saline, dissected in about 1-mm^3 fragments, placed on a nylon mesh, and cultured for 2 weeks in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Cells migrating from the explants were collected by trypsinization, suspended in DMEM, plated in 25-cm^2 flasks, and cultured in the same medium at 37°C. At confluence, the cells were detached with 0.1% trypsin/EDTA, counted, and plated at the density indicated below to evaluate parameters of cell proliferation and differentiation. The cell cultures were previously shown to display osteoblastic characteristics *in vitro*, such as ALP activity, synthesis of the osteoblast specific protein osteocalcin, and responsiveness to calcitropic hormones.[19,20]

Mutation Analysis

Mutation analysis in osteoblastic cells isolated from patients 1 and 3 were performed at the Metabolic Diseases Branch of the National Institute of Diabetes and Digestive and Kidney Diseases (National Insti-

tutes of Health, Bethesda, MD; Drs. A. Shenker and A. Spiegel) as described.[6,9,13] Briefly, a short fragment of the $G_s\alpha$ gene containing the Arg^{201} codon was amplified with polymerase chain reaction (PCR), and mutations were identified with allele-specific oligonucleotide hybridization. This technique measures the specific binding of short, radioactive-labeled oligonucleotide probes that either match the wild-type DNA sequence exactly (R201) or contain a single-base substitution (R201H or R201C). Mutations were confirmed by restriction digestion analysis.[13] In patient 1, mutation analysis was also performed on DNA isolated from normal osteoblastic cells isolated from a noninvolved area. For patient 3, osteoblastic cells obtained in a normal patient were used as a negative control. The mutation analysis in this patient has been previously reported.[13]

In patient 2, mutation analysis was conducted by direct sequencing in osteoblastic cells isolated from the affected bone lesion and from a normal bone in a control patient as follows. Genomic DNA was extracted from osteoblastic cell cultures using the Qiagen (Courtaboeuf, France) DNA extraction kit, as recommended by the manufacturer. One hundred nanograms of genomic DNA was amplified in 100 μl of reaction buffer containing 0.1 mmol/L deoxynucleotide triphosphates, 2.5 mmol/L $MgCl_2$, upstream (5′-GCGCTGTGAACACCCCACG-3′) and downstream (5′-GAATGTCAAGAAACCATGATC-3′) oligonucleotide primers (30 pmol each), and 2 U of *Taq* DNA polymerase (Eurobio, Les Ulis, France) to yield a single product spanning codon 201. Amplification consisted of denaturation at 95°C for 5 minutes followed by 30 cycles (95°C for 1 minute, 50°C for 2 minutes, and 72°C for 2 minutes). Negative controls were included in every PCR. A second round PCR was done with 2 μl of products from the first round in the presence of 0.5 U of *Taq* polymerase for another 30 cycles (94°C for 45 seconds, 55°C for 1 minute, and 72°C for 30 seconds). The PCR products were analyzed on a 1.5% agarose gel and G_s corresponding bands (353 bp) were purified by the Wizard purification system (Promega, Madison, WI). The purified PCR products were subjected to direct DNA sequencing (fmol sequencing kit; Promega) using ^{33}P-labeled PCR primers.

Osteoblastic Cell Proliferation

The time course of cell growth was evaluated in an osteoblastic cell culture obtained from each subject by incorporation of [^{3}H]thymidine into DNA.[19,20] Cells plated at 10,000 cells/cm^2 in 24-well plates were cultured in DMEM with 10% FCS. Cell growth was studied for 1 to 3 days in patients 1 and 3 and for up to 14 days in patient 2. Every 2 days, the cells were labeled with 2 μCi/well [^{3}H]thymidine, and incorporation into DNA was determined 24 hours later. At this time point, the cell layer was collected by trypsinization, DNA was precipitated with trichloracetic acid, the trichloracetic acid-insoluble fraction was dissolved in NaOH, and [^{3}H]thymidine incorporation into DNA was measured in three aliquots by liquid scintillation.

Osteoblastic Cell Differentiation

ALP activity and osteocalcin production, two markers of osteoblast differentiation,[21] were determined in each osteoblastic cell culture, as described.[19,20] Osteoblastic cells were plated at 10,000 cells/cm^2 in six-well plates and cultured in DMEM with 10% FCS until confluence. The medium was removed, and the cells were cultured in the presence of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) or its solvent. A dose-response curve was performed in cells derived from patient 2, whereas cells from patients 1 and 3 were treated with an optimal dose of $1,25(OH)_2D$ (10 nmol/L) in serum-free medium. After 48 h of treatment, the medium was removed and frozen for osteocalcin determination. Cells were rinsed with cold phosphate-buffered saline, scraped in distilled water, and sonicated. ALP activity in the cell lysate was determined by a colorimetric method using phenyl phosphate as substrate (BioMerieux, Marcy l'Etoile, France). The protein content of the cell lysate was determined colorimetrically (Bio-Rad, Ivrys/Seine, France). The activity of the enzyme was expressed as nanomoles of *p*-nitrophenol released per minutes per milligram of protein. Osteocalcin concentration in the medium was measured in duplicate by radioimmunoassay (Cis-Oris, Gif sur Yvette, France) using a specific antibody raised against bovine osteocalcin. The maximum intraassay and interassay coefficients of variation for the range of concentrations evaluated were 3.7 and 6.6%, respectively, and the lower detection limit of the assay was 0.35 μg/liter. The production of osteocalcin in the medium was evaluated in each culture of individual patients and was corrected for cell protein.

cAMP Production

The basal intracellular cAMP levels and the response to human (1–34)-parathyroid hormone (PTH) was assessed in osteoblastic cells in patient 1 with MAS. Mutated osteoblastic cells and normal cells from the same patient were plated at 10,000 cells/cm^2 in six-

well plates and cultured in DMEM with 10% FCS until confluence. The cells were then incubated in DMEM supplemented with 1% FCS and 0.1 mmol/L isobutylmethylxanthine (Sigma Chemical Co., St. Louis, MO), an inhibitor of phosphodiesterases. After a 1-hour incubation at 37°C, the cells were treated for 10 minutes with 20 nmol/L PTH (Sigma) or its solvent. The cells were then placed on ice, scraped, and sonicated in the medium, and the intracellular cAMP concentrations were determined by radioimmunoassay (Immunotech, Luminy, France).[19,20]

Statistical Analysis

The data are expressed as the mean ± SEM and were analyzed using the statistical package super-ANOVA (Macintosh; Abacus Concepts, Inc., Berkeley, CA). Differences between values in normal and affected areas in the same subjects were evaluated by Students' *t*-test with a minimal significance of $P < 0.05$.

Results

Mutation Analysis in Osteoblastic Cells

In patient 1 with MAS, the mutation analysis with PCR and allele-specific oligonucleotide hybridization showed the presence of an R201C mutation in osteoblastic cells isolated from the dysplastic area (Figure 1A). The fact that the signals produced by the wild-type and mutant probes were of equal intensity indicates that all of the sampled osteoblastic cells carry the mutant allele, and the abnormal genome contained both mutant and wild-type alleles. Mutation analysis of osteoblastic cells isolated from a noninvolved bone in the same patient showed hybridization with the wild-type R201 oligoprobe only, showing that the mutation was absent in normal osteoblastic cells (Figure 1A).

Mutation analysis in patient 2 with MAS showed that the R201C mutation (substitution of Cys for Arg) was detected in osteoblastic cells isolated from dysplastic bone lesions, whereas the mutation was absent in normal bone from a control patient (Figure 1B).

We previously reported that the R201H mutation (substitution of His for Arg) was present in osteoblastic cells isolated from the involved area and was absent in normal cells from a noninvolved area in patient 3 with monostotic fibrous dysplasia.[13] As expected for a somatic mutation, the mutations were found only in DNA from osteoblastic cells isolated from abnormal bone but not in peripheral DNA from the same patients.[13] These results confirm the presence of an activating mutation in the gene encoding the α-subunit of G_s in osteoblastic cells isolated from involved areas in monostotic fibrous dysplasia and MAS.

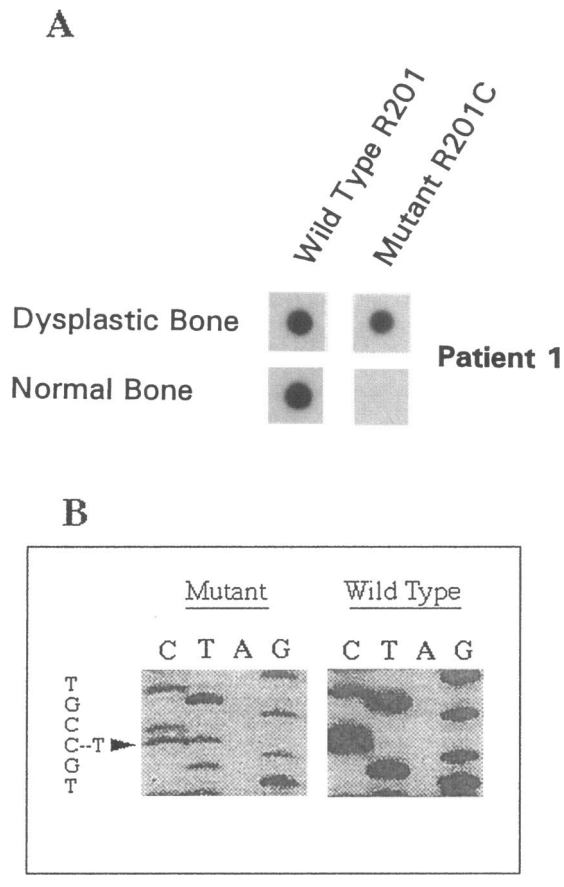

Figure 1. A: *Analysis of DNA with allele-specific oligonucleotide hybridization showing the mutation of $G_s\alpha$ with a substitution of cysteine for arginine (R201C) in osteoblastic cells from patient 1 with MAS and the absence of mutation in osteoblastic cells from a normal bone (wild type). PCR products amplified from osteoblastic cells were cross-linked to nylon membranes and hybridized with ^{32}P-labeled wild-type R201 and mutant R201C oligonucleotide probes.* **B:** *Direct sequence analysis of the amplified genomic fragment spanning exons 7 and 8 of $G_s\alpha$ gene in osteoblastic cells from dysplastic bone lesion in patient 2 with MAS. The direct sequence of PCR products reveals a heterozygous single Cys to Thr substitution of the first base of codon 201, with a change from Arg to Cys. The mutation was absent in osteoblastic cells from a normal subject (wild type).*

Bone Formation in Bone Lesions Expressing the $G_s\alpha$ Mutation

Examination of undecalcified bone sections of involved areas in patients 1 and 2 with MAS and in patient 3 with monostotic fibrous dysplasia showed characteristics of fibrotic areas and woven bone. The dysplastic bone was characterized by the presence of woven immature bone and extensive marrow fibrosis (Figure 2a). There were few mature osteo-

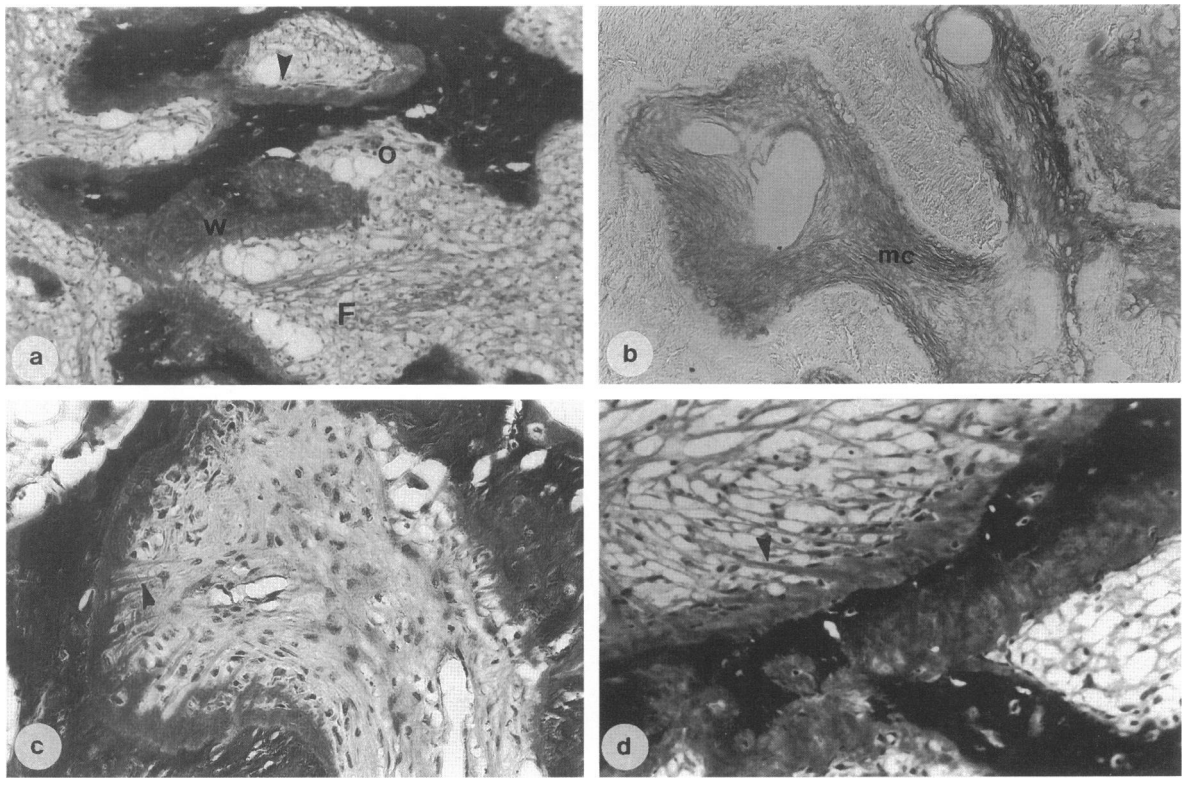

Figure 2. *Photomicrographs of representative sections of a dysplastic bone lesion (patient 1 with MAS). The dysplastic bone was characterized by woven (w) immature bone and extensive marrow fibrosis (F), few morphologically mature osteoblasts* (arrowhead), *and osteoclasts (o) along the bone surface* (a) *(Goldner Trichrome staining, magnification ×125). Histochemical staining for ALP showed numerous immature ALP-positive mesenchymal (mc) cells* (b) *(magnification ×250). These cells were depositing numerous collagenous fibers along the bone surface* (c *and* d, arrowheads) *(Goldner Trichrome staining, magnification ×250).*

blasts along the bone surface, and some areas presented osteoclasts and bone resorption (Figure 2a). Most cells in the marrow stroma stained for ALP, an early marker of osteoprogenitor cells (Figure 2b), and these mesenchymal cells were actively depositing collagenous matrix along the bone surface (Figure 2, c and d). The same characteristics of fibrous dysplasia were found in the less severely affected area in patient 2 (Figure 3a). Examination of the more severely affected area in the same patient showed the presence of more numerous mesenchymal cells and extensive fibrotic tissue with some early mesenchymal condensation but no calcified bone (Figure 3b), showing that this area was less mature and had more proliferating cells than the less severely affected area.

The histomorphometric analysis indicated that most, but not all, parameters of bone formation were

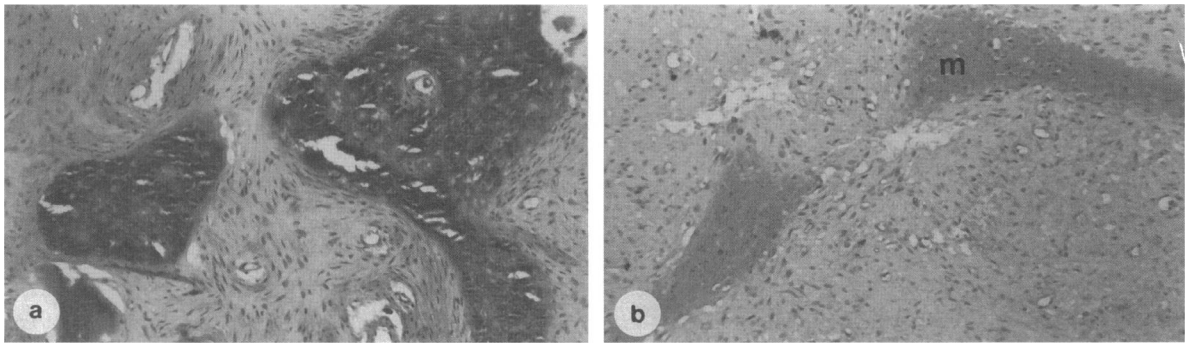

Figure 3. *Histological aspect of undecalcified bone sections of two dysplastic areas in patient 2 with MAS, showing numerous mesenchymal cells and extensive fibrotic tissue, early mesenchymal (m) condensation, and no calcified tissue in the more severely affected area* (b), *indicating an immature phenotype compared with the less severely affected area* (a). *(Goldner Trichrome staining, magnification ×250).*

Table 1. *Histomorphometric Analysis of Involved Areas in Monostotic Fibrous Dysplasia (FD) and Polyostotic Fibrous Dysplasia Associated with MAS*

	Osteoid surface (% bone surface)	Osteoblast surface (% osteoid surface)	Osteoclast surface (% bone surface)	Osteoclast number (/mm^2)	Woven bone (% bone volume)
Patient 1 (MAS)	94.9 ± 3.9	9.4 ± 1.6	1.8 ± 0.5	1.5 ± 0.2	80 ± 5
Patient 2 (MAS)	97.0 ± 0.2	4.6 ± 0.8	4.0 ± 0.1	2.2 ± 0.1	100 ± 0
Paitent 3 (monostotic FD)	88.7 ± 0.2	11.0 ± 1.8	3.1 ± 0.5	1.6 ± 0.2	100 ± 0

Data are the mean ± SEM of three to four values.

elevated in monostotic or polyostotic fibrous dysplasia (Table 1). The osteoid surface and the amount of woven bone were markedly elevated, showing increased matrix synthesis. In contrast, the fraction of osteoid surface with morphologically mature osteoblasts (osteoblast surface) was very low (Table 1) compared with the normal values (37.5 ± 4.9%) found in age-matched normal bone.[22] These histological findings show that there was a low number of differentiated osteoblasts along the bone surface, whereas numerous ALP-positive mesenchymal cells were actively synthesizing a woven bone matrix in both MAS and monostotic fibrous dysplasia.

Proliferation of Osteoblastic Cells Expressing the $G_s\alpha$ Mutation

Examination of osteoblastic cell proliferation revealed clear differences between cells expressing the $G_s\alpha$ mutation and normal cells in the same patient. In patient 1 with MAS, the growth of osteoblastic cells isolated from dysplastic bone and expressing the mutation was twofold greater at 1 and 3 days of culture than in normal cells, as evidenced by [^{3}H]thymidine incorporation into DNA (Figure 4A). In patient 2 with MAS, the proliferation of osteoblastic cells expressing the mutation in the more severely affected lesion was twofold higher than in the less severely affected area at 1 to 14 days of culture (Figure 5). In patient 3 with monostotic fibrous dysplasia, the growth of mutated osteoblastic cells isolated from the dysplastic bone was also twofold greater at 1 and 3 days of culture than in normal osteoblastic cells obtained from adjacent bone in the same patient (Figure 4B). These data indicate that the $G_s\alpha$ mutation is associated with an increased proliferation of osteoblastic cells compared with normal cells in both MAS and monostotic bone fibrous dysplasia and suggest that the increased proliferation of mesenchymal cells expressing the mutation correlates with the severity of the fibrotic lesion.

Differentiation of Osteoblastic Cells Expressing the $G_s\alpha$ Mutation

ALP activity and osteocalcin production, early and late markers of osteoblast differentiation, respectively,[21] were evaluated in osteoblastic cells expressing

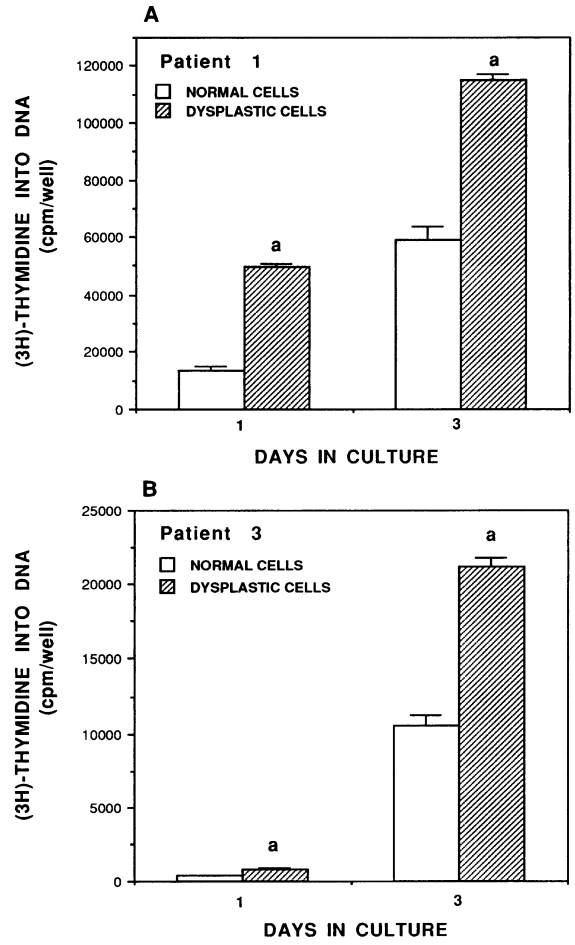

Figure 4. *Osteoblastic cells expressing the $G_s\alpha$ mutation in patient 1 with MAS* (A) *and in patient 3 with monostotic fibrous dysplasia* (B) *showed increased cell proliferation, as evaluated by DNA synthesis, compared with normal osteoblastic cells isolated from a noninvolved adjacent area in the same patient* (a: $P < 0.001$ versus *normal cells*).

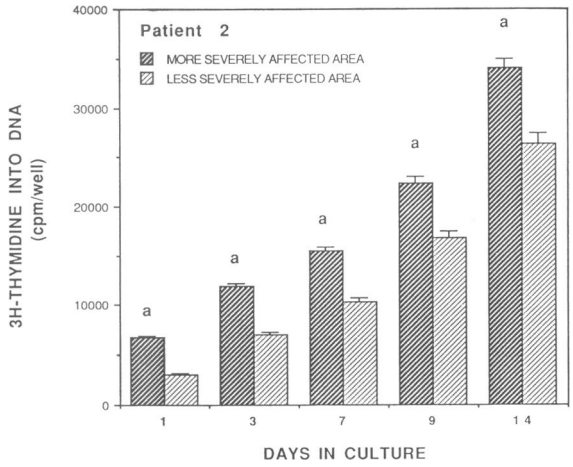

Figure 5. *Osteoblastic cells expressing the $G_s\alpha$ mutation in the more severe fibrotic dysplastic area in patient 2 with MAS showed increased* in vitro *proliferation, as evaluated by DNA synthesis, compared with mutated cells from the less severely affected area* (a: $P < 0.001$ versus *less severe area*).

the $G_s\alpha$ mutation in comparison with cells isolated from normal areas in the same patient. In patient 1 with MAS, the basal and $1,25(OH)_2D$-stimulated ALP activity were similar in mutated and normal cells (Figure 6A). In patient 2 with MAS, ALP activity was also found to be similar in osteoblastic cells isolated from the more severely and less severely affected areas in basal conditions and after stimulation with $1,25(OH)_2D$ (Figure 7A). In contrast to ALP activity, the basal osteocalcin production was lower in osteoblastic cells expressing the $G_s\alpha$ mutation compared with normal cells in patient 1 with MAS (Figure 6B). The response to 10 nmol/L $1,25(OH)_2D$, however, was not different in normal and dysplastic cells from this patient (Figure 6B). Moreover, in patient 2 with MAS, osteocalcin production was lower in osteoblastic cells isolated from the more severe fibrotic area compared with the less severely affected area in the basal condition and after stimulation with $1,25(OH)_2D$ (Figure 7B). These findings suggest that ALP activity per cell is not affected in mutated cells and does not correlate with the severity of the disease, whereas osteocalcin, a late marker of osteoblast differentiation, is decreased in osteoblastic cells expressing the mutation and is reduced in mutated osteoblastic cells present in the more severely affected bone lesion.

The evaluation of intracellular cAMP accumulation in osteoblastic cells isolated from patient 1 showed that the basal cAMP level was about 30% higher in dysplastic cells expressing the $G_s\alpha$ mutation compared with normal cells from the same patient (Figure 8), indicating that the cAMP production was increased in osteoblastic cells expressing the

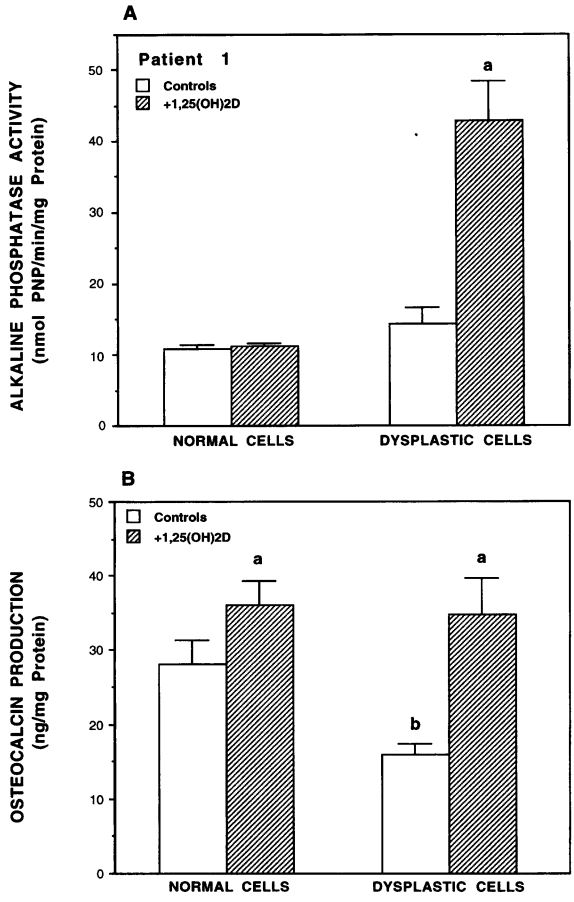

Figure 6. *Osteoblastic cells expressing the $G_s\alpha$ mutation in patient 1 with MAS showed normal basal and 10 nmol/L (48 hours) $1,25(OH)_2D$-stimulated ALP activity* (A) *and decreased osteocalcin production* (B) *compared with normal cells isolated from a noninvolved adjacent area in the same patient.* (a: $P < 0.001$ versus *controls;* b: $P < 0.01$ versus *normal cells*).

mutation. Treatment with 10 nmol/L (1–34)-PTH increased the intracellular cAMP production by 200 to 300%, and the response to PTH was found to be similar in mutated and normal cells in this patient (Figure 8). These *in vitro* findings indicate that osteoblastic cells expressing the $G_s\alpha$ mutation in dysplastic bone lesions have an increased intracellular cAMP levels associated with an increased proliferation rate, reduced osteocalcin production, and normal responsiveness to calcitropic hormones *in vitro*.

Discussion

Previous studies have shown that bone lesions in MAS[9,11] and osteoblastic cells derived from monostotic lesions[13] present an activating mutation of $G_s\alpha$ at Arg^{201}, suggesting that the mutation in bone-forming cells may be responsible for the abnormal bone formation in fibrous dysplastic lesions. In this study,

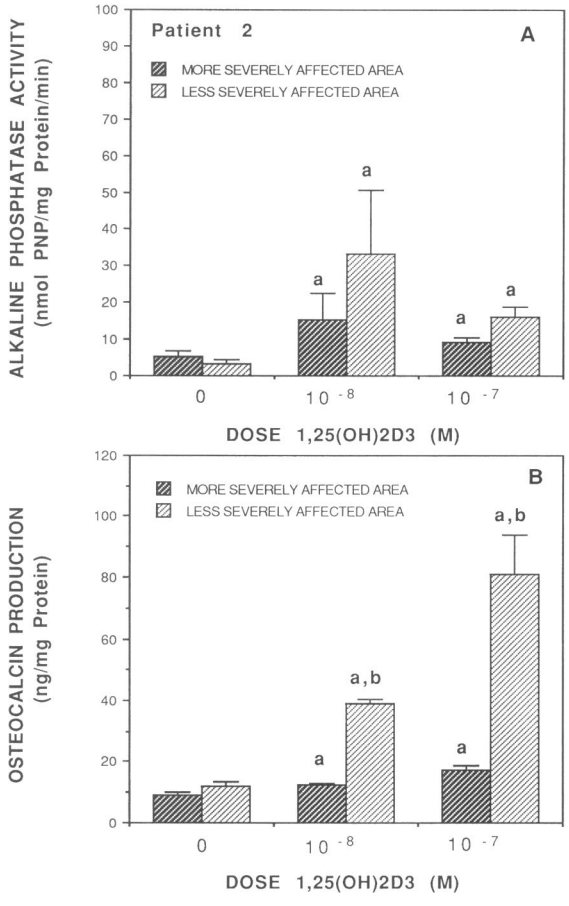

Figure 7. *Effect of 1,25$(OH)_2D$ (48 hours) on ALP activity* (A) *and osteocalcin production* (B) *in osteoblastic cells expressing the $G_s\alpha$ mutation in two lesions of patient 2 with MAS. ALP levels were similar and osteocalcin production was lower in osteoblastic cells isolated from the more severe fibrotic area compared with the less severely affected dysplastic area in this patient in either basal or 10 nmol/L (48 hours) 1,25$(OH)_2D$-stimulated conditions* (a: $P < 0.05$ versus *controls;* b: $P < 0.01$ *compared with less severe lesion*).

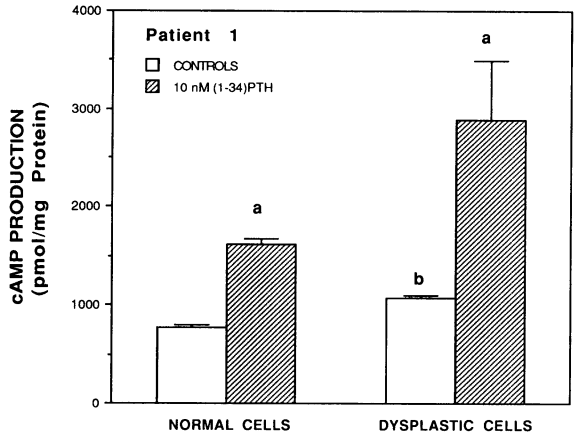

Figure 8. *Osteoblastic cells expressing the $G_s\alpha$ mutation in patient 1 with MAS showed increased basal intracellular cyclic AMP levels compared with normal cells isolated from a noninvolved adjacent area in the same patient, with a normal response to 20 nmol/L human (1–34)-PTH* (a: $P < 0.001$ versus *controls;* b: $P < 0.001$ versus *normal cells*).

we show that the expression of an activating $G_s\alpha$ mutation in osteoblastic cells is associated with an increased cell proliferation and inappropriate cell differentiation, resulting in an increased number of immature mesenchymal cells and deposition of a fibrotic matrix in monostotic fibrous dysplasia and MAS.

In both monostotic and polyostotic fibrous dysplasia, the histomorphometric analysis showed that the bone lesions were characterized by an extensive deposition of woven bone matrix, indicating a high rate of bone matrix formation. A very low number of morphologically mature osteoblasts were present, whereas numerous ALP-positive cells were found to actively deposit collagen fibers along the bone surface, showing that the excessive collagenous matrix in dysplastic bone results from increased deposition of fibrotic matrix by immature osteoblastic cells.

Previous studies from our laboratory have shown that human osteoblastic cell cultures are potent tools to investigate the pathophysiology of osteoblast dysfunctions.[20,22] In the present study, we used osteoblastic cell cultures to investigate the cellular abnormalities in dysplastic bone cells expressing the $G_s\alpha$ mutation. Osteoblastic cells isolated from dysplastic bone expressed the Arg^{201} mutation of $G_s\alpha$, whereas normal osteoblastic cells from noninvolved areas in the same patients did not express the mutation, which corroborates our previous data in osteoblastic cells from patients with monostotic fibrous dysplasia.[13] We now show that, in both monostotic and polyostotic fibrous dysplasia, DNA synthesis in osteoblastic cells expressing the $G_s\alpha$ mutation was higher than in normal osteoblastic cells isolated from an adjacent area in the same patients. In addition, the proliferation of mutated osteoblastic cells was higher in the area showing the more fibrotic characteristics, indicating that the increased growth of mutated osteoblastic cells correlated with the severity of the fibrotic lesion. This is consistent with the observation in soft tissues that the highest proportion of mutant alleles was found in regions of apparently increased cell proliferation in MAS.[6]

We then determined whether the activating $G_s\alpha$ mutation is associated with osteoblastic cell dysfunction. We found that ALP activity, an early marker of the osteoblast phenotype, was similar in mutated osteoblastic cells from dysplastic areas and in paired normal cells. In contrast, the production of osteocalcin, a late marker of osteoblast differentiation,[21] was lower in cells expressing the $G_s\alpha$ mutation compared with normal cells from the same pa-

tient. In addition, osteocalcin production by osteoblastic cells was lower in the more severe fibrotic lesion than in the less severely affected area, indicating that the increased growth in mutated osteoblastic cells was associated with reduced cell differentiation in the affected bone lesions.

We also found that the mutation was associated with increased basal cAMP production in osteoblastic cells from MAS compared with normal cells from the same patient. Intracellular cAMP levels, however, were not strikingly increased, possibly because of a compensatory rise in cAMP phosphodiesterase activity[14,23] or unstability of the mutant protein. These data suggest that the somatic missense mutation in the $G_s\alpha$ gene in osteoblastic cells leads to constitutively activated adenylate cyclase activity, elevated cAMP levels, and increased proliferation of hormonally responsive osteoblastic cells, which resulted in overproduction of a disorganized collagenous matrix. The polyostotic and isolated monostotic fibrous dyplasia may thus result from abnormal proliferation, and perhaps reduced differentiation, of mesenchymal osteoblast progenitor cells that harbor an activating mutation of $G_s\alpha$. These findings in mutated osteoblastic cells from dysplastic bone are consistent with the observation that an overactive cAMP-signaling pathway stimulates the growth of tissues, such as the gonads, thyroid, adrenal cortex, and melanocytes, in patients with MAS.[7,8,11] In a subset of pituitary somatotroph tumors, mutation at either Arg^{201} or Gln^{227} was also found to activate adenylate cyclase activity, resulting in increased cell growth.[3–5] The increased osteoblastic cell proliferation in fibrous dysplasia may thus be directly related to the elevated cAMP levels resulting from the activating $G_s\alpha$ mutation. Agents such PTH raise cAMP production and increase the production of insulin-like growth factor I, which stimulates osteoblastic cell proliferation.[24] In addition, bone features found in tertiary hyperparathyroidism induced by autonomous hypersecretion of PTH in humans are similar to those found in MAS.[25] These observations suggest that the increased intracellular cAMP content resulting from $G_s\alpha$ overactivity may induce increased proliferation and inappropriate differentiation of bone-forming cells, leading to the formation of an immature fibrotic matrix. This is consistent with a recent study indicating that the reduction of $G_s\alpha$ protein expression in another cell type is associated with induction of cell differentiation.[26] Recently, it was reported that interleukin 6 secretion is increased in cultured fibrous cells with a $G_s\alpha$ mutation in fibrous dysplasia from two patients with MAS,[27] which may in part account for the increased bone resorption reported in bone lesions of MAS.[27] However, increased interleukin 6 production is unlikely to be responsible for the observed increased osteoblastic cell proliferation in MAS, because interleukin 6 does not affect human osteoblastic cell growth *in vitro*.[28]

Several intracellular events may be involved in the cAMP pathway signaling induced by the activating $G_s\alpha$ mutation. Increased intracellular cAMP concentration results in activation of the catalytic subunit of protein kinase-A, which phosphorylates cAMP response element-binding proteins acting as potent *trans*-activating factors.[29] cAMP response element-binding proteins may thus be constitutively activated in osteoblastic cells expressing the $G_s\alpha$ mutation, as found in human somatotroph adenomas.[30] On the other hand, the constitutive activation of $G_s\alpha$ may lead to stimulate several early immediate genes, including c-*fos* and c-*jun*, via a protein kinase A-mediated pathway, as found in pituitary cells.[31,32] In bone, the protooncogene c-*fos* seems to play a critical role in the control of normal osteoblastic cell growth and differentiation *in vivo* and *in vitro*.[33–35] In addition, increased expression of c-*fos* was recently found in bone lesions in patients with fibrous dysplasia.[36] These observations suggest that the $G_s\alpha$ mutation may stimulate osteoblastic cell proliferation by inducing chronic stimulation of early immediate genes. Our finding that activating mutation of $G_s\alpha$ in osteoblastic cells leads to constitutive activation of adenylate cyclase, increased cell proliferation, and inappropriate cell differentiation will serve as a basis for the development of studies to elucidate the functional target genes of the $G_s\alpha$ mutation in osteoblasts, which results in the overproduction of a disorganized fibrotic bone matrix in polyostotic and monostotic fibrous dysplasia.

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References

1. Albright F, Butler AM, Hampton AO, Smith P: Syndrome characterized by osteitis fibrosa disseminata, areas of pigmentation and endocrine dysfunction, with precocious puberty in females: report of five cases. N Engl J Med 1937, 216:727–746

2. McCune DJ: Osteitis fibrosa cystica: the case of a nine year old girl who also exhibits precocious puberty, multiple pigmentation of the skin and hyperthyroidism. Am J Dis Child 1936, 52:743–747
3. Landis CA, Harsh G, Lyons J, Davis RL, McCormick F, Bourne HR: Clinical characteristics of acromegalic patients whose pituitary tumors contain mutant G_s protein. J Clin Endocrinol Metab 1990, 71:1416–1420
4. Lyons J, Landis CA, Harsh G, Vallar L, Grunewald K, Feichtinger H, Duh Q-Y, Clark OH, Kawasaki E, Bourne HR, McCormick F: Two G protein oncogenes in human endocrine tumors. Science 1990, 249:655–659
5. Vallar L, Spada A, Giannattasio G: Altered G_s, and adenylate cyclase activity in human GH-secreting pituitary adenomas. Nature 1987, 330:566–568
6. Weinstein LS, Shenker A, Gejman PV, Merino MJ, Friedman E, Spiegel AM: Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. N Engl J Med 1991, 325:1688–1695
7. Spiegel AM, Weinstein LS, Shenker A: Abnormalities in G protein-coupled signal transduction pathways in human disease. J Clin Invest 1993, 92:1119–1125
8. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L: GTPase inhibiting mutations activate the α chain of G_s and stimulate adenylyl cyclase in human pituitary tumours. Nature 1989, 340:692–696
9. Shenker A, Weinstein LS, Sweet DE, Spiegel AM: An activating $G_s\alpha$ mutation is present in fibrous dysplasia of bone in the McCune-Albright syndrome. J Clin Endocrinol Metab 1994, 79:750–755
10. Malchoff CD, Reardon G, MacGillivray DC, Yamase H, Rogol AD, Malchoff DM: An unusual presentation of McCune-Albright syndrome confirmed by an activating mutation of the G_s α-subunit from a bone lesion. J Clin Endocrinol Metab 1994, 78:803–806
11. Masters SB, Miller RT, Chi MH, Chang FH, Beiderman B, Lopez NG, Bourne HR: Mutations in the GTP-binding site of GS α alter stimulation of adenylyl cyclase. J Biol Chem 1989, 264:15467–15474
12. Grabias SL, Campbell CJ: Fibrous dysplasia. Orthop Clin North Am 1977, 8:771–783
13. Shenker A, Chanson P, Weinstein LS, Chi P, Spiegel AM, Lomri A, Marie PJ: Osteoblastic cells derived from isolated lesions of fibrous dysplasia contain activating somatic mutations of the $G_s\alpha$ gene. Hum Mol Genet 1995, 4:1675–1676
14. Zachery I, Masters SB, Bourne HR: Increased mitogenic responsiveness of Swiss 3T3 cells expressing constitutively active $G_s\alpha$. Biochem Biophys Res Commun 1990, 168:1184–1193
15. Spiegel AM, Shenker A, Weinstein LS: Receptor-effector coupling by G proteins: implications for normal and abnormal signal transduction. Endocr Rev 1992, 13: 536–562
16. Chanson P, Dib A, Visot A, Derome PJ: McCune-Albright syndrome, and acromegaly: clinical studies and responses to treatment in five cases. Eur J Endocrinol 1994, 131:229–234
17. Hott M, Marie PJ: Glycol methacrylate as an embedding medium for bone tissue. Stain Technol 1987, 62: 51–57
18. Baron R, Vignery A, Neff L, Silverglate A, Maria AS: Processings of undecalcified bone specimens for bone histomorphometry. Bone Histomorphometry. Edited by RR Recker. Boca Raton, FL, CRC Press Inc., 1983, pp 13–36
19. Marie PJ, Lomri A, Sabbagh A, Baslé M: Culture and behavior of osteoblastic cells isolated from normal trabecular bone surfaces. In Vitro Cell & Dev Biol 1989, 25:373–380
20. Marie PJ, de Vernejoul MC, Connes D, Hott M: Decreased DNA synthesis by cultured osteoblastic cells in eugonadal osteoporotic men with defective bone formation. J Clin Invest 1991, 88:1167–1172
21. Rodan GA, Rodan SB: Expression of the osteoblastic phenotype. Bone and Mineral Research, vol 2. Edited by WA Peck. Amsterdam, Elsevier, 1990, pp 244–285
22. Marie PJ: Human osteoblastic cells: a potential tool to assess the etiology of pathologic bone formation. J Bone Miner Res 1994, 9:1847–1850
23. Ma YH, Landis C, Tchao N, Wang J, Rodd G, Hanahan D, Bourne HR, Grodsky GM: Constitutively active stimulatory G-protein α_s in β-cells of transgenic mice causes counterregulation of the increased adenosine 3′,5′-monophosphate and insulin secretion. Endocrinology 1994, 134:42–47
24. McCarthy TL, Thomas MJ, Centrella M, Rotwein P: Regulation of insulin-like growth factor I transcription by cyclic adenosine 3′,5′-monophosphate (cAMP) in fetal rat bone cells through an element within exon I: protein kinase A-dependent control without a consensus AMP response element. Endocrinology 1995, 136: 3901–3908
25. Rasmussen H, Bordier P: The Physiological and Cellular Basis of Metabolic Bone Disease. Baltimore, Williams & Wilkins, 1974
26. Meißner JD, Brown GA, Mueller WH, Scheibe RH: Retinoic acid-mediated decrease of $G_{\alpha s}$ in the differentiation of HL-60 myeloid cells. Exp Cell Res 1996, 225: 112–121
27. Yamamoto T, Ozono K, Kasayama S, Yoh K, Hiroshima K, Takagi M, Matsumoto S, Michigamai T, Yamaoka K, Kishimoto T, Okada S: Increased IL-6 production by cells isolated from the fibrous bone dysplasia tissues in patients with McCune-Albright syndrome. J Clin Invest 1996, 98:30–35
28. Modrowski D, Godet D, Marie PJ: Involvement of interleukin 1 and tumour necrosis factor α as endogenous growth factors in human osteoblastic cells. Cytokine 1995, 7:720–726
29. de Groot RP, Sassone-Corsi P: Hormonal control of gene expression: multiplicity and versatility of cyclic adenosine 3′,5′-monophosphate-responsive nuclear regulators. Mol Endocrinol 1993, 7:145–153
30. Bertherat J, Chanson P, Montminy M: The cyclic aden-

osine 3′,5′-monophosphate-responsive factor CREB is constitutively activated in human somatotroph adenomas. Mol Endocrinol 1995, 9:777–783

31. Gaiddon C, Boutillier AL, Monnier D, Mercken L, Loeffler JP: Genomic effects of the putative oncogene Gαs. Chronic transcriptional activation of the c-*fos* proto-oncogene in endocrine cells. J Biol Chem 1994, 269, 36:22663–22671
32. Gaiddon C, Tian J, Loeffler JP, Bancroft C: Constitutively active Gs α-subunits stimulate Pit-1 promoter activity via a protein kinase A-mediated pathway acting through deoxyribonucleic acid binding sites both for Pit-1 and for adenosine 3′,5′-monophosphate response element-binding protein. Endocrinology 1996, 137:1286–1291
33. Grigoriadis, AE, Schellander K, Wang ZK, Wagner EF: Osteoblast are target cells for transformation in c-*fos* transgenic mice. J Cell Biol 1993, 122:685–701
34. Machwate M, Jullienne A, Moukhtar M, Marie PJ: Temporal variation of c-*fos* proto-oncogene expression during osteoblast differentiation and osteogenesis in developing rat bone. J Cell Biochem 1995, 57:62–70
35. Machwate M, Jullienne A, Moukhtar M, Lomri A, Marie PJ: c-*fos* protooncogene is involved in the mitogenic effect of transforming growth factor-β in osteoblastic cells. Mol Endocrinol 1995, 9:187–198
36. Candeliere GA, Glorieux FH, Prud'homme J, St-Arnaud R: Increased expression of the c-*fos* proto-oncogene in bone from patients with fibrous dysplasia. N Engl J Med 1995, 332:1546–1551